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(54) Title: NUCLEIC ACID CHELATE CONJUGATE AS THERAPEUTIC AND DIAGNOSTIC AGENTS

(57) Abstract

Novel conjugates are provided comprising, in particular, stable nucleotide sequences joined to a chelating agent. The conjugates find use in inhibiting expression of mRNA, in killing pathogens, and in providing selection for particular classes of cells or killing particular classes of cells. Polyamine, polycarboxylic acid, chelating agents are employed in the conjugates as exemplary.

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NUCLEIC ACID CHELATE CONJUGATE
AS THERAPEUTIC AND DIAGNOSTIC AGENTS

INTRODUCTION

Technical Field

The field concerns the use of modified oligo-
nucleotides as therapeutic agents, inhibiting matura-
tion or expression of transcription products _in vivo_
and _in vitro_.

Background

The biological revolution has introduced a
variety of new techniques resulting in the ability to
determine various cellular and subcellular processes.
As the understanding has increased as to how the cell
maintains its viability and proliferates, new opportu-
nities have opened for utilizing novel therapeutic
approaches. One technique which has been used in a
variety of ways in the laboratory and is being expanded
from the laboratory into real life situations, is the
use of anti-sense sequences to modulate the fate of a
transcription product in a host cell.

For the most part, the approaches in employing
anti-sense sequences have been twofold. In one
approach, cells in culture are modified by injection of
a large excess of a DNA sequence which is complementary
to the sequence of a mRNA present in the cell. A sub-
stantial reduction in the expression product is
observed. In another approach, one can introduce a
transcription cassette comprising a promoter functional
in the host and a DNA sequence which results in the
production of a mRNA which is complementary to an endo-
genous mRNA. Again, one observes a reduction in the
expression product to which the transcription construct
is directed.

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If, however, anti-sense sequences are to
become a useful therapeutic agent, there are many pro-
blems and difficulties to be overcome. As a therapeu-
tic agent, a method must be found which allows for the
5   transfer of the anti-sense sequence across the cellular
membrane. The anti-sense sequence must be designed so
as to be relatively stable to degradation, particularly
by nucleases. In addition, there remain concerns about
the specificity of the sequence, particularly where one
10 does not wish to kill the host cell. Also of concern
is how to reach the necessary concentration in the cell
to provide the desired level of inhibition of tran-
scription product maturation or expression. Finally,
any technique which is devised for fulfilling the above
15 objectives, must take into consideration such problems
as toxicity, immunogenicity, solubility, and the like.
There is, therefore, substantial interest in being able
to develop anti-sense sequences which will be effective
as therapeutic agents in the modulation of the forma-
20 tion of mRNA and its expression.

Relevant Literature

Anti-sense nucleic acid sequences have been
reported to selectively block translation of a number
25 of mRNAs (Izant and Weintraub, Cell (1984) 36:1007-
1015; Izant and Weintraub, Science (1985) 229:345-352;
Melton, Proc. Natl. Acad Sci. USA (1985) 82:144-148;
Mizuno et al., Proc. Natl. Acad. Sci. USA (1984)
81:1966-1970). Short oligonucleotides have been
30 reported to provide for high selectivity. (Wallace et
al., Nucl. Acids Res. (1979) 6:3543-3557; Wallace et
al., Nucl. Acids Res. (1981) 9:879-894; Smith, et al.,
Proc. Natl. Acad. Sci. USA (1986) 83:2787-2791;
Szostak, et al., Methods Enzymol. (1979) 68:419-429; Wu
35 et al., Prog. Nucl. Acid Res. and Mol. Biol. (1978)
21:102). The short oligonucleotides are able to
rapidly and specifically bind to specific target

sequences. (Itakura and Riggs, Science (1980) 209:
1401; Szostak et al., Methods Enzymol. (1979) 68:419-
429; Noyes et al., J. Biol. Chem. (1979) 254:7472-7475;
Noyes et al., Proc. Natl Acad. Sci. USA (1979) 76:1770-
5   1774; Agarwal et al., J. Biol. Chem. (1981) 256:1023-
1028; Tullis et al., Biochem. Biophys. Res. Comm.
(1980) 93:941). See also the use of oligonucleotide
probes to detect point mutations. (Orkin et al., J.
Clin. Invest. (1983) 71:775; Conner et al., Proc. Natl.
10   Acad. Sci. USA (1983) 80:278; Piratsu et al., New Eng.
J. Med. (1983) 309:284-287; Wallace et al., Nucl.
Acids. Res. (1981) 9:879-894). The rapidity with which
synthetic DNAs hybridize is related to the complexity
of the probe. (Wetmur and Davidson, J. Mol. Biol.
15   (1967) 31:349; Wallace et al., Nucl. Acids Res. (1979)
6:3543-3557; Tullis et al., Biochem. Biophys. Res.
Comm. (1980) 93:941; Meinkoth and Wahl, Anal. Biochem.
(1984) 138:267-284).

        Both normal and phosphorous modified oligonu-
20   cleoides have been reported to selectively block the
expression of specific RNAs. Zamecnik and Stephenson,
Proc. Natl. Acad. Sci. USA (1978) 75:280-284; Tullis et
al., J. Cellular Biochem. Suppl. (1984) 8A:58
(Abstract); Kawasaki, Nucl. Acids Res. (1985) 13:4991;
25   Haeptule et al., Nucl. Acids Res. (1986) 14:1427-1448;
Walder et al., Science August 1, 1986; Stephenson and
Zamecnik, Proc. Natl. Acad. Sci. USA (1978) 75:285-288
Cornelissen et al., Nucl. Acids Res. (1986) 14:5605-
5614; Minshall and Hunt, Nucl. Acids Res. (1986)
30   14:6433-6451).

        Protection from nuclease degradation can be
achieved by employing phosphotriesters and methylphos-
phonates. (Barrett et al., Biochemistry (1974) 13:
4897-4906; Miller et al., Biochemistry (1977) 16:1988-
35   1997; Miller et al., Biochemistry (1981) 20:1873-1880;
Jayaraman et al., Proc. Natl. Acad. Sci. USA (1981)
77:1537-1541; Blake et al., Biochemistry (1985)

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24:6132-6138; Blake et al., Biochemistry (1985) 24:
6139-6145; Smith et al., Proc. Natl. Acad. Sci. USA
(1986) 83:2787-2791; Agris et al., Biochemistry (1986)
25:6268-6275; Miller et al., Nucl. Acids. Res. (1983)
5   11:6225-6242).

       A number of groups have tried to enhance the
binding affinity by modifying the nucleotide sequence.
(Summerton, J. Theor. Biol. (1978) 78:77-99; Knorre et
al., Adv. Enz. Reg. (1984) pp. 277-300; Vlassov et al.,
10   Adv. Enz. Reg. (1986) pp. 301-320).

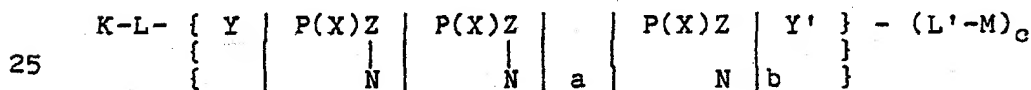   SUMMARY OF THE INVENTION
       Oligonucleotide conjugates are provided, where
a specific sequence of at least eight nucleotides is
15   covalently linked to an ion chelating group and option-
ally to other groups to enhance transport across the
cell membrane. The resulting compositions are found to
effectively block the function of a sequence comple-
mentary to the oligonucleotide. The compositions find
20   use as agents in vivo and in vitro for modulating
intracellular transcription product maturation or
expression.

             DESCRIPTION OF THE SPECIFIC EMBODIMENTS
25   Methods and compositions are provided for
modulating transcriptional maturation or expression by
employing oligonucleotide conjugates. The oligonucleo-
tide conjugates have at least two components: an oli-
gonucleotide sequence of at least eight nucleotides;
30   and a chelating agent. In addition, other groups may
be present which include a linker joining the chelating
agent to the oligonucleotide sequence, a hydrophobic
group for enhancing the transport across the membrane,
or other moieties to enhance binding affinity, reduce
35   toxicity, enhance solubility, or other characteristics
of interest.

The subject compositions will generally have a
hybridizing sequence, namely a polynucleotide unit from
about 8 to 30, more usually from about 8 to 20, prefer-
ably from about 12 to 18 members. The molecular weight
5   will normally be under about 10 kD, usually under about
6 kD, although high molecular weight molecules may be
used in special circumstances. The chelating agent may
be any one of a large number of chelating agents which
are able to chelate metal ions capable of acting as
10   scissile and/or free radical initiating agents, by
themselves or in conjunction with other compounds which
may be present in the host cell or introduced in the
host cell. The chelating agent will be able to chelate
one of a variety of metals, such as iron, cobalt,
15   nickel, molybdenum, vanadium, or other metal ion which
may be encountered in the cytoplasm of the cell and may
serve to initiate the formation of free radicals,
resulting in the scission or other modification of the
transcription product, preventing its normal function
20   in the host cell.

For the most part, the compositions of the
subject invention will have the following formula:

$$K-L-\left\{ Y \; \left| \; \begin{matrix} P(X)Z \\ | \\ N \end{matrix} \; \right| \; \left[\begin{matrix} P(X)Z \\ | \\ N \end{matrix} \right]_a \; \left| \; \begin{matrix} P(X)Z \\ | \\ N \end{matrix} \right|_b \; Y' \right\} - (L'-M)_c$$

25

Wherein:

K represents the chelating agent capable of
chelating a metal ion, which ion is capable of catalyz-
30   ing a chemical reaction in the physiological medium of
the cytoplasm of a cell, which results in a chemical
transformation of mRNA inhibiting expression, particu-
larly degradative modification;

L is a bond or linking unit derived from a
35   polyvalent functional group having at least one atom,
which functional group may be of from about 1 to 20
atoms other than hydrogen, comprising carbon, nitrogen,

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oxygen, sulfur, and phosphorous, where the linking
group may be aliphatic, aromatic, alicyclic, hetero-
cyclic, or combinations thereof; substituted or unsub-
stituted; generally having from 0 to 10 heteroatoms,
5   usually from 0 to 6 heteroatoms, where the cyclic com-
pounds will usually have from 1 to 2 rings, usually 1
ring, and the aliphatic groups may be branched,
straight chained, heterosubstituted or unsubstituted;
desirably the linking unit will have a chain of 2 to
10   20, usually 4 to 16 atoms normally free of linkages
capable of enzymatic degradation; L may be joined
through Y to the terminal phosphorous or may be joined
at any convenient site of the oligonucleotide chain,
being linked to P, C, N, O or S of the base (N),
15   saccharide (Z), or group linked to phosphorous (X);

         X is usually a pair of electrons, alkyl of
from 1 to 3 carbon atoms, chalcogen (oxygen or sulfur),
or amino, particularly NH;

         Z is a monosaccharide, particularly of 5 to 6
20   carbon atoms, more particularly of 5 carbon atoms,
which may have from 0 to 1 hydroxyl groups replaced by
hydrogen, and will usually be substituted by phospho-
rous at the 2, 3, 5, or 6 positions, particularly at
the 3 and 5 positions, and substituted at the one posi-
25   tion, by the purine or pyrimidine, where the sugars may
include such sugars as ribose, arabinose, xylylose
glucose, galactose, deoxy, particularly 2-deoxy,
derivatives thereof, etc.;

         L' is a linker group which is derived from a
30   polyvalent functional group having at least one atom,
and not more than about 60 atoms other than hydrogen,
usually not more than about 30 atoms other than hydro-
gen, having up to about 30 carbon atoms, usually not
more than about 20 carbon atoms, and up to about 10
35   heteroatoms, more usually up to about 6 heteroatoms,
particularly chalcogen, nitrogen, phosphorous, etc.;

M is a moiety, particularly imparting amphi-
philic properties to the compound, a hydrophobic or
amphiphilic moiety which will have a ratio of carbon to
heteroatom of at least about 2:1, usually at least
5   about 3:1, frequently up to greater than about 20:1,
may include hydrocarbons of at least 6 carbon atoms and
not more than about 30 carbon atoms, polyoxy compounds
(alkyleneoxy), where the oxygen atoms are joined by
from about 2 to 10 carbon atoms, usually 2 to 6 carbon
10   atoms, preferably 2 to 3 carbon atoms, and there will
be at least about 6 units and usually not more than
about 200 alkyleneoxy units, more usually not more than
about 100 units, generally not more than about 60
units;
15   Y is a bond to L or a terminal group; Y' is a
bond to L', linking L' to a terminal phosphorous, or a
terminal group; when a terminal group, Y and Y' are
oxy, thio, amino or substituted functionalities
thereof, e.g., oxyether, alkylamino, etc. or alkyl of
20   up to about 20, usually of up to about 6 carbon atoms,
Y and Y' usually not being more than about 20 carbon
atoms, more usually being not more than about 10 carbon
atoms;
            N is any natural or unnatural base (purine or
25   pyrimidine), capable of binding to and hybridizing with
a natural purine or pyrimidine, where N may be adenine,
cytidine, thymidine, guanidine, uracil, orotidine,
inosine, etc.;
            a is at least 4, usually at least 5, and not
30   more than about 50, usually not more than about 35;
            b and c are each 0 or 1.
            The functional groups which find use with the
linking groups, L and L', include functionalities such
as oxy, non-oxo-carbonyl (carboxy carbonyl), oxo-
35   carbonyl (aldehyde or ketone), the nitrogen or sulfur
analogs thereof, e.g. imino, thiono, thio, amidino,
etc., disulfide, amino, diazo, hydrazino, oximino,
phosphate, phosphono, etc.

The linking group to the hybridizing sequence
may be linked through an oxygen or sulfur present on a
pyrimidine, purine, sugar or phosphorous group, to a
carbon atom of a pyrimidine or purine, or to a phos-
5   phorous atom.   The links may be ethers to oxygen and
sulfur, esters, both organic and inorganic, to oxygen
and sulfur, amides, both organic and inorganic, to
amines and phosphorous, and alkylamino to amino groups.
Esters include carboxylates, e.g. carboxy esters,
10   carbamates, carbonates, etc., and phosphates, phos-
phonates, etc.   Of particular interest is linking at
the terminal unit of the hybridizing sequence through a
sugar hydroxyl, particularly at the 5'-position.

            The phosphorous moiety may include phosphates,
15   phosphoramidates, phosphordiamidate, phosphorothioate,
phosphorothionate, phosphorothiolate, phosphoramidothi-
olate, phosphonates, phosphorimidate, and the like.
Where the phosphorous is bound to other than oxygen of
the sugar, the sugar will be modified by having an
20   amino or thio functionality at the site of binding, so
that both amino and thio sugars may be employed to
provide for novel linkages between the phosphorous and
the sugar.

            K is a chelating agent, having at least 3
25   heteroatoms, which are oxygen, nitrogen, or sulfur,
usually combinations thereof, more usually having about
6 heteroatoms or more, which may serve to chelate a
metal ion capable of acting to inactivate, particularly
to enhance cleavage, of a nucleic acid.   The function-
30   alities may include carbonyl, oxy, thiono, amino,
amido, mercapto, thioether, imino, where carbonyl oxy-
gens will normally be separated by at least 2 carbon
atoms, usually up to 6 carbon atoms, more usually up to
4 carbon atoms; except for amido, heteroatoms will nor-
35   mally be separated by at least 2 carbon atoms.   Conven-
iently, there will be at least 2 non-oxo-carbonyl
groups frequently at least 3 non-oxo-carbonyl groups

and not more than about 6, usually not more than about
5 non-oxo-carbonyl groups. Of particular interest are
alkylene diamines and polyalkylene diamines having from
3 to 8, usually 4 to 6 carboxyl groups, usually as
5　carboxymethylene groups, e.g.,
$R_2N(CH_2)_mN(T)((CH)_nN(J))_x(CH_2)_pNR_2$, wherein R is a
carboxyalkylene group of from 2 to 3 carbon atoms or H,
at least one R on each N being carboxyalkylene, m, n
and p are the same or different and are 2 to 4, usually
10　2 to 3, and x is 0 to 2.

Illustrative chelating groups include ethy-
lenediaminetetraacetic acid, dipropyleneaminepenta-
acetic acid, diethylenetriaminepentaacetic acid, 2,3-
bis-(2'-acetamidoethyl)succinic acid, porphyrins,
15　phthalocyanins tetraacetic acid, and crown ethers.

A wide variety of linking groups may be
employed, depending upon the nature of the terminal
nucleotide, the functionality selected for, whether the
linking group is present during the synthesis of the
20　oligonucleotide, the functionality present on the
hydrophobic moiety and the like. A number of linking
groups are commercially available and have found exten-
sive use for linking polyfunctional compounds. The
linking groups include: $-OCH_2CH_2NHCO(CH_2)_nCONH-;-$
25　$OCH_2CH_2NH-X-(CH_2)_nNH-;-O-P(O)(OH)NHCO(CH_2)_nCOHN-;-$
$OCH_2CH_2NHCO\phi S-;-NH(CH_2)_nNH;-O(CH_2)_nO-;-O(CH_2CH_2NH)_m-;-$
$NH(CH_2)_nSYN; -CO(CH_2)_nCO; -SCH_2CH_2CO-; -CO\phi NYS-;$
$-(NCH_2CH_2)_mCH_2N-;$ charged and uncharged homo- and
copolymers of amino acids, such as polyglycine, polyly-
30　sine, polymethionine, etc. usually of about 500 to
2,000 daltons; wherein O is phenyl; X is 2,5-
quinondiyl, Y is S-(3-succindoyl) to form succinimidyl,
n is usually in the range of 2 to 20, more usually 2 to
12, and m is 1 to 10, usually 1 to 6.
35　The amphiphilic character imparting or solu-
bility modifying group (M) may be a wide variety of
groups, being aliphatic, aromatic, alicyclic, heterocy-

clic, or combinations thereof, substituted or unsubsti-
tuted, usually of at least 6, more usually at least 12
and not more than about 1000, usually not more than
about 500, more usually not more than about 200 carbon
5   atoms, having not more than about 1 heteroatom per 2
carbon atoms, being charged or uncharged, including
alkyl of at least 6 carbon atoms and up to about 30
carbon atoms, usually not more than about 24 carbon
atoms, fatty acids of at least about 6 carbon atoms,
10   usually at least about 12 carbon atoms and up to about
24 carbon atoms, glycerides, where the fatty acids will
generally range from about 12 to 24 carbon atoms, there
being from 1 to 2 fatty acids, usually the 2 or 3
positions or both, aromatic compounds having from 1 to
15   4 rings, either mono- or polycyclic, fused or unfused,
polyalkyleneglycols where the alkylenes are of from 2
to 8, usually of from 2 to 4 carbon atoms, more usually
2 to 3 carbon atoms, there usually being at least about
6 units more usually at least about 10 units, and
20   usually fewer than about 500 units, more usually fewer
than about 200 units, preferably fewer than about 100
units, where the alkylene glycols may be homopolymers
or copolymers; alkylbenzoyl, where the alkyl group will
be at least about 6 carbon atoms, usually at least
25   about 10 carbon atoms, and not more than about 20
carbon atoms; alkyl phosphates or phosphonates, where
the alkyl group will be at least 6 carbon atoms, usu-
ally at least about 12 carbon atoms and not more than
about 24 carbon atoms, usually not more than about 20
30   carbon atoms, or the like.

        The "M" group may be charged or uncharged,
preferably being uncharged. Illustrative groups
include polyethylene glycol having from about 40 to 50
units, copolymers of ethylene and propylene glycol,
35   laurate esters of polyethylene glycols, triphenyl-
methyl, naphthylphenylmethyl, palmitate, distearyl-
glyceride didodecylphosphatidyl, cholesteryl, arachi-
donyl, octadecanyloxy, tetradecylthio, etc.

Functionalities which may be present include oxy, thio, carbonyl, (oxo or non-oxo), cyano, halo, nitro, aliphatic unsaturation, etc.

In designing the nucleic acid sequence, it
5   will be desirable to have a high affinity between the subject composition and the target single stranded nucleic acid sequence. Sequences will preferably be selected having greater than 40% GC content, more preferably greater than 50% and may have 60% or more GC
10 content. For optional selectivity, the melting temperature of the hybrid to be formed should be 5 to 10°C above the ambient temperature at which the hybrid forms, usually the ambient temperature being 37°C in a mammalian host. For mammalian hosts, the melting
15 temperature will generally be chosen to be about 42-50°C. The target sequence should be selected to be relatively free of secondary and tertiary structure. In many mRNA's, an open region will be present in the vicinity of the start codon (AUG).
20   In preparing the subject compositions, various strategies may be employed, depending upon whether "M" is present, the nature of "M", the nature of the oligo-nucleotide and the nature of the linking group. Thus, so long as care is taken that the addition of the two
25 different groups, "M" and the chelating group, do not interfere with one another, the groups may be added sequentially.

One technique for providing the chelating agent may be found in Dryer and Dervan, supra. In this
30 technique, a modified nucleoside is employed during the synthesis of the oligonucleotide. Thymidine may be modified at the methyl group by providing for a carboxy alkyl group. The carboxy group may then be further functionalized with an alkylene diamine, and the amino
35 group employed for amide formation with a carboxy containing chelating agent. The modified thymidine may then be employed as a nucleotide reagent in the auto-mated synthesis of the oligonucleotide.

Alternatively, the final nucleotide adduct in
the synthesis of the oligonucleotide may be functional-
ized in a variety of ways which may serve to act as a
linking unit to the chelating agent. For example,
5   after removal of the trityl protective group an amino-
ethanolphosphoramidite is added, as described by the
supplier (Applied Biosystems, Foster City, CA) to pro-
vide for an available amino group. After deblocking
and removing the oligonucleotide chain from the sup-
10   port, the amino group is then available for linking to
the chelating agent. Alternatively, the oligonucleo-
tide is phosphorylated employing a polynucleotide
kinase, followed by formation of a phosphoramidate
using an activating agent, such as 1-methylimidazole or
15   a water soluble carbodiimide, in the presence of an
alkylene diamine, providing for an amino functionality
(Chu and Orgel, DNA (1985) 4:327-331). A further
alternative is to deblock the oligonucleotide while
retaining the oligonucleotide on the support, followed
20   by treatment with carbonyldiimidazole. After removal
of excess of the carbonyldiimidazole, a diamine may be
added to provide an aminoalkylcarbamate (Wachter et
al., Nucl. Acids Res. (1986) 14:7985-7994).

Where "M" is to be added, a mercaptan group
25   may be provided as part of the functionalizing agent or
separate from the functionalizing agent. The mercaptan
group may be part of the linker to the support or may
be part of the functionalizing agent of the oligo-
nucleotide, where both the chelating agent and "M" may
30   be bound to the same linking group. Besides mercaptan
groups, maleimido groups may be employed, where "M" or
the chelating agent may have a mercaptan group to form
a thioether.

Various active functionalities can be employed
35   to produce a covalent linkage, such as isocyanates,
isothiocyanates, diazo groups, imino chlorides, imino
esters, anhydrides, acylhalides, sulfinylhalides,

sulfonyl chlorides, etc. Conditions for carrying out
the various reactions and joining non-nucleotide
moieties to nucleotide moieties may be found in Chu and
Orgel DNA (1985) 4:327-331; Smith et al. Nucl. Acids
5   Res. (1985) 13:2399-2412.

      The linking arms, "M", and the chelating
moiety may be added at various times, depending upon
the particular reaction scheme. For the most part, the
chelating agent may be part of a nucleoside and be
10 included in the synthesis of the oligonucleotide or may
be added after oligonucleotide formation. "M" will
normally be added after oligonucleotide formation.

      For the most part, reaction conditions will be
mild and will employ polar solvents or combinations of
15 polar and nonpolar solvents. Solvents will vary and
include water, acetonitrile, dimethylformamide, diethyl
ether, methylene chloride, dimethylsulfoxide, etc.
Reaction conditions will be for the most part in the
range of about -100-60°C. Usually, after completion of
20 the reaction between components of the conjugate, the
resulting product will be subjected to purification.

      The manner of purification may vary, depending
upon whether the oligonucleotide is bound to a support.
For example, where the oligonucleotide is bound to a
25 support, after addition of the linking arm to the
oligonucleotide, unreacted chains may be degraded, so
as to prevent their contaminating the resulting
product. Where the oligonucleotide is no longer bound
to the support, whether only reacted with the linking
30 arm or as the conjugate to the chelating agent or as
the final product, each of the intermediates or final
product may be purified by conventional techniques,
such as electrophoresis, solvent extraction, HPLC,
chromatography, or the like. The purified product is
35 then ready for use.

The subject products will be selected to have
an oligonucleotide sequence complementary to a sequence
of interest. The sequence of interest may be present
in a prokaryotic or eukaryotic cell, a virus, a normal

5   or neoplastic cell. The sequences may be bacterial
sequences, plasmid sequences, viral sequences, chromo-
somal sequences, mitochondrial sequences, plastid
sequences, etc. The sequences may involve open reading
frames for coding proteins, ribosomal RNA, snRNA,

10   hnRNA, introns, untranslated 5'- and 3'-sequences
flanking open reading frames, etc. The subject
sequences may therefore be involved in inhibiting the
availability of an RNA transcript, inhibiting expres-
sion of a particular protein, enhancing the expression

15   of a particular protein by inhibiting the expression of
a repressor, reducing proliferation of viruses or neo-
plastic cells, etc.

The subject conjugates may be used in culture
or in vivo for modifying the phenotype of cells, limit-

20   ing the proliferation of pathogens such as viruses,
bacteria, protista, mycoplasma, or the like, or induc-
ing morbidity in neoplastic cells or specific classes
of normal cells. Thus, one can use the subject composi-
tions in therapy, by administering to a host subject in

25   a diseased state, one or more of the subject composi-
tions to inhibit the transcription and/or expression of
the native genes of a cell. The subject compositions
may be used for protection of a mammalian host from a
variety of pathogens, e.g., enterotoxigenic bacteria,

30   Pneumococcus, Neisseria, etc.; protists, such as
Giardia, Entamaeba, etc.; neoplastic cells, such as
lymphoma, leukemia, carcinoma, sarcoma etc.; specific
B-cells, specific T-cells, such as helper cells,
supressor cells, CTL, NK, etc.

35   The subject compositions will be selected so
as to be capable of inactivating sequences of interest,
particularly mRNA, or in some circumstances the subject

composition can be used with other nucleic acid
moieties, e.g., tRNA, snRNA, DNA, e.g., plasmids,
viruses, etc. Thus, the subject compositions may bind
to mRNA and provide for cleavage of the mRNA, so as to
5   prevent the expression of a product. By employing
sequences which are relatively inert to degradation,
the lifetime of the chelate conjugate may be substan-
tially extended in the host cell, so as to have a rela-
tively high kill ratio per sequence.
10   The subject sequences may be complementary to
such sequences as sequences expressing growth factors,
lymphokines, immunoglobulins, T-cell receptor sites,
MHC antigens, DNA or RNA polymerases, antibiotic resis-
tance, multiple drug resistance (mdr), genes involved
15   with metabolic processes, in the formation of amino
acids, nucleic acids, or the like, DHFR, etc. as well
as introns or flanking sequences associated with the
open reading frames.
         The subject compositions may be administered
20   to a host in a wide variety of ways, depending upon
whether the compositions are used in vitro or in vivo.
In vitro, the compositions may be introduced into the
nutrient medium, so as to modulate expression of a par-
ticular gene by transferring across the membrane into
25   the cell interior such as the cytoplasm and nucleus.
The subject compositions may find particular use in
protecting mammalian cells in culture from mycoplasma,
for modifying phenotype for research purposes, for
evaluating the effect of variation of expression on
30   various metabolic processes, e.g., production of parti-
cular products, variation in product distribution, or
the like. While no particular additives are necessary
for transport of the subject compositions intracellu-
larly, the subject compositions may be modified by
35   being encapsulated in liposomes or other vesicle, may
be used in conjunction with permeabilizing agents,
e.g., non-ionic detergents, Sendai virus, etc.

For _in vivo_ administration, depending upon its
particular purpose, the subject compositions may be
administered in a variety of ways, such as injection,
infusion, tablet, etc., so that the compositions may be
5   taken orally, parenterally, intravascularly, intraperi-
toneally, subcutaneously, intralesionally, or the
like. The compositions may be formulated in a variety
of ways, being dispersed in various physiologically
acceptable media, such as deionized water, water, phos-
10 phate buffered saline, ethanol, aqueous ethanol, formu-
lated in the lumen of vesicles, microencapsulated, etc.
Because of the wide variety of applications
and manners of administration, no particular composi-
tion can be suggested. Rather, as to each indication,
15 the subject compositions may be tested in conventional
ways and the appropriate concentrations determined
empirically. Other additives may be included, such as
stabilizers, buffers, additional drugs, detergents,
etc. These additives are conventional, and would
20 generally be present in less than about 5 wt%, usually
less than 1 wt%, being present in an effective dosage,
as appropriate. For fillers or excipients, these may
be as high as 99.9% of the composition, depending upon
the amount of active material necessary.
25 The following examples are presented by way of
illustration not by way of limitation.

EXAMPLE 1
Synthesis of Diethylenetriamine pentacetic acid
30   (DTPA) Conjugated Oligomers
Chemical Synthesis of DNA. The chemical syn-
thesis of DNA was carried out using slight modifica-
tions of the conventional phosphoramidite methods on an
Applied Biosystems (Model 381) DNA synthesizer. The
35 method used is a modification of the technique
described by Caruthers and coworkers (Beaucage and
Caruthers, 1984, European Patent Application
82/102570).

17

In this technique, nucleoside phosphoramidites
dissolved in anydrous acetonitrile are mixed with
tetrazole and sequentially coupled to the 5' hydroxy
terminal nucleotide of the growing DNA chain bound to
5   controlled pore glass (CPG) supports via a succinate
spacer (Matteucci and Caruthers, Tetrahedron Letters
(1980) 21:719-722). Nucleoside addition is followed by
capping of unreacted 5' hydroxyls with acetic
anhydride, iodine oxidation, and 5' detritylation in
10   trichloroacetic acid-methylene chloride. The resin
bound oligomer is then dried by extensive washing in
anhydrous acetonitrile and the process repeated.
Normal cycle times using this procedure are 12 minutes
with condensation efficiencies of >98% (as judged by
15   trityl release).

Chemical Synthesis of Amine Linker Arm
Containing DNA Oligonucleotides Bound to CPG Glass
Beads. The completed fully blocked DNA chains can
subsequently be modified to contain a 5' amino linker
20   arm while still attached to the CPG support. Several
methods have been used to accomplish this.

Aminolink Procedure for 5' Amine Linker Arm
Attachment. At the end of the synthesis, trityl is
removed from the product oligonucleotide chains and an
25   aminoethanolphosphoramidite is added to the 5' hydroxyl
using the Aminolink procedure developed and marketed by
Applied Biosystems (Foster City, CA). The resin bound
oligonucleotide is then deblocked and released from the
column using a method appropriate to the type of phos-
30   phate linkage present. For normal phosphodiesters,
hydrolysis in concentrated ammonia is appropriate. For
DNA triesters and methylphosphonates, ethylene diamine
(EDA) phenol deblocking followed by ammonia or EDA:
ethanol release is appropriate. Results indicate that
35   all of the cyanoethyl-phosphorus and aryl-amide base
blocking groups are removed under these conditions.

Addition of Amine Containing Linker Arms Using
Phosphoramidate Linkage. One alternative is the use of
the technique of Chu and Orgel, Proc. Natl. Acad. Sci.
USA (1986) 82:963-967 to add linker arms to the 5' end
5 of any oligonucleotide. In this instance, the oligo-
nucleotide is first phosphorylated using polynucleotide
kinase. After purification by polyacrylamide gel
electrophoresis, the product DNA containing a free 5'-
hydroxyl is phosphorylated with the forward reaction of
10 T4 polynucleotide kinase according to Chu and Orgel,
supra (1986). Phosphorylated oligomers are separated
from unreacted ATP using a C-18 reverse phase column
(Waters SEP-PAK) according to the direction of the
manufacturer. The phosphorylated oligomer is then
15 treated with 0.1M 1-methyl imidazole, 0.1M 1-ethyl-3-
(3-dimethylaminopropyl) carbodiimide, 0.1M alkyl
diamine (e.g., hexane diamine), pH 7, under conven-
tional conditions in an aqueous medium to form the
desired phosphoramidate containing a free amine with
20 the following structure

$$\text{oligomer} - \overset{\overset{\text{O}}{\|}}{\underset{\underset{\text{OH}}{|}}{\text{P}}} - \text{NH} - (\text{CH}_2)_n - \text{NH}_2$$
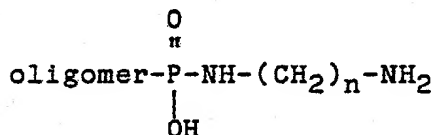
25

Addition of An Amine Linker Arm Using Carbonyl
Diimidazole (CDI). A third alternative to the addition
of an amine linker arm to the 5' end of any oligo-
30 nucleotide is to use carbonyl-bis (imidazole). In this
technique, the CPG bound, base blocked oligomer (trityl
off) is first treated with CDI (50 mg/ml) in dry aceto-
nitrile for 30 to 45 minutes to form the imidazole
carboxylic acid ester. The excess CDI is then washed
35 off with acetonitrile and water and the diamine of
choice added to the column generally in a mixture of
acetonitrile or dioxane and water. The diamine forms a

stable carbamate linkage after a brief incubation.   The
oligomer can then be deblocked and released from the
column under conditions appropriate to the type of
phosphate linkage present.

5   Attachment of the Cleavage Unit DTPA.   Once
the amine terminated oligomer is deblocked and charac-
terized, the cleavage unit was added using the follow-
ing method.   Ten units of the oligomer were dissolved
in 100 µl dimethylformamide containing 0.1 M DTPA (bis-
10   anhydride).   The mixture was incubated for 1-2 hours at
room temperature, and the excess DTPA removed by gel
filtration and concentration on Centricon C10 mem-
branes.   The final product was dried in vacuo and dis-
solved to a final concentration of 1 mM in water (based
15   on its optical density at 260nM) and stored frozen.
This solution was stable for at least 1 month.   The
compound was homogeneous as judged by polyacrylamide
gel electrophoresis.

20   EXAMPLE 2
          Synthesis of DTPA Derivatives of Normal DNAs Using
             Imidazole Activated Carboxylic Acid Esters and
                        Long Chain Aminoalkanes
                In this example, a 20 nucleotide DNA comple-
25   mentary to the initiation region of mouse beta globin
mRNA was synthesized according to the method given in
EXAMPLE 1.   After synthesis, the product material was
retained on the synthesis support with trityl removed
from the 5' end of the molecule.   The solid material
30   was then thoroughly washed with anhydrous acetonitrile
and blown dry under a stream of dry argon.   Using a
plastic syringe, 1 cc of 0.3M carbonyldiimidazole dis-
solved in anhydrous acetonitrile was pushed slowly
through the synthesis column containing the support
35   bound oligomer over a period of 45 minutes.   The 5'
carbonylimidazole activated oligomer on the column was
then washed free of excess reagent with 15 ml of aceto-

nitrile and then treated with 0.2 M decanediamine in
acetonitrile:water (10:1) for 30 minutes.

The material on the column was washed free of
unreacted decane-diamine with acetonitrile and water,
5   and then eluted from the column in concentrated ammon-
ium hydroxide. After removal from the column, the
ammonium hydroxide solution containing the oligomer
conjugate was placed in a sealed vial and incubated 5
hours at 55°C.

10   The product is then lyophilized several times
from 50% aqueous ethanol and purified via reversed
phase HPLC C-8 silica columns eluting 5 to 50% aceto-
nitrile/25 mM ammonium acetate, pH 6.8 in a linear
gradient. If required, the material was further puri-
15   fied by ion-exchange HPLC on Nucleogen DEAE 60-7 elut-
ing 20% acetonitrile/25 mM ammonium acetate, pH 6.5.
The recovered product is then characterized by gel
electrophoresis in 15% polyacrylamide gels carried out
as described by Maxam and Gilbert (Meth. Enzymol.
20   (1980) 68:499-560). Oligonucleotides in finished gels
are visualized using Stains-all.

As a further check on the material, the
presence of a primary amine can be determined by two
methods. First, reaction with fluorescamine produced a
25   fluorescent product characteristic of the presence of a
primary amine while no fluorescence is observed with
similarly treated control oligomers of the same type,
but lacking the amine linker. Second, the decane-con-
jugate was dissolved in 100 μl 0.1 M sodium bicarbonate
30   to which was added 1 mg of fluorescein isothiocyanate.
After 1 hour of incubation, the unreacted FITC was
removed by gel filtration chromatography on Sephadex
G-25 spun columns. The product was then analyzed by
polyacrylamide gel electrophoresis as described above,
35   and the fluorescent band product visualized under UV
illumination. A single fluorescent band is observed
which corresponds to the oligomer visualized by subse-
quent staining with Stains-all.

The product of this reaction is an aminoalkyl
carbamate coupled to the 5' end of the oligonucleotide.
The alkylcarbamate is stable to moderate exposure to
concentrated base. The free amino group distal to the
carbamate linkage is available for subsequent deriva-
tion which can be accomplished according to the method
given in EXAMPLE 1. The structure of the final con-
jugate synthesized by this method is illustrated as:

$$\text{oligomer} - O - \overset{\overset{\displaystyle O}{\|}}{C} - NH - (CH_2)_{10} - NH - \overset{\overset{\displaystyle O}{\|}}{C} - DTPA$$
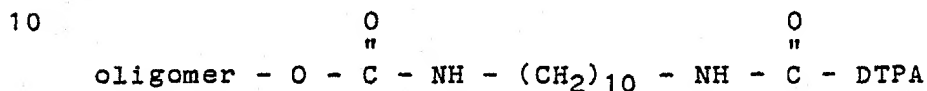
EXAMPLE 3
Synthesis of DTPA Derivatives of DNAs Using
Imidazole Activated Carboxylic Acid Esters
and a Poly-D-lysine Linker

In this example, a 25 nucleotide DNA comple-
mentary to the initiation region of rabbit beta globin
mRNA was synthesized according to the method given in
EXAMPLE 1. After synthesis, the CPG support containing
the oligomer was treated with 80% acetic acid for 30
minutes to remove trityl from the 5' end of the mole-
cule. The solid material was then thoroughly washed
with anhydrous acetonitrile and blown dry under a
stream of dry argon and treated with 0.3M CDI as in
EXAMPLE 4. The 5' CDI activated oligomer on the column
was then washed free of excess reagent with 15 ml of
acetonitrile and then treated with 0.2 M poly-D-lysine
(MW+1,000) dissolved in 50% acetonitrile containing
0.1 M sodium phosphate, pH 8 for 16 hours at room
temperature.

The material on the column was washed free of
salts and unreacted polylysine with water and aceto-
nitrile and then eluted from the column in concentrated
ammonium hydroxide. After removal from the column, the
ammonium hydroxide solution containing the oligomer

conjugate was incubated for 5 hours at 55°C in a sealed
glass vial. The product was then lyophilized several
times from 50% aqueous ethanol and purified via gel
filtration chromatography on TSK G4000SW in 100 mM Tris
5 buffer, pH 7.5. The presence of a primary amine was
determined by reaction with fluorescamine. No fluores-
cence was observed with control oligomers lacking the
polyamine linker.

The polyamine conjungate cannot easily be
10 characterized by gel electrophoresis since it is elec-
trostatically and molecularly polydisperse. In order
to render the polyamine conjugate negatively charged,
the complex was reacted with FITC to label the molecule
and to neutralize the positive charges on the amines.
15 This was accomplished by dissolving a portion of the
material in 100 μl 0.1 M sodium bicarbonate to which
was added 1 mg of fluoresceinisothiocyanate. After 1
hour of incubation, the unreacted FITC was removed by
gel filtration chromatography on Sephadex G-25 spun
20 columns (Maniatis et al., Molecular Cloning - A
Laboratory Manual, Cold Spring Harbor Lab., Cold Spring
Harbor, New York (1982)). The product was then
analyzed by polyacrylamide gel electrophoresis carried
out as described by Maxam and Gilbert (Meth. Enzymol.
25 (1980) 68:499-560) and the fluorescent band product
visualized under UV illumination. A single broad
fluorescent band is observed which corresponds to the
DNA visualized by Stains-all.

The structure of this conjugate may be
30 illustrated as having the general formula:

$$
\text{oligomer} - O - \overset{\overset{\textstyle O}{\|}}{C} - NH - (\underset{\underset{\textstyle NH}{\underset{\textstyle |}{(CH_2)_4}}}{\overset{|}{CH}} - \overset{\overset{\textstyle O}{\|}}{C} - NH)_n - \overset{\overset{\textstyle O}{\|}}{C} - \underset{\underset{\textstyle NH_2}{\underset{\textstyle |}{(CH_2)_4}}}{\overset{|}{CH}} - COOH
$$

35

$$O = C - DTPA$$
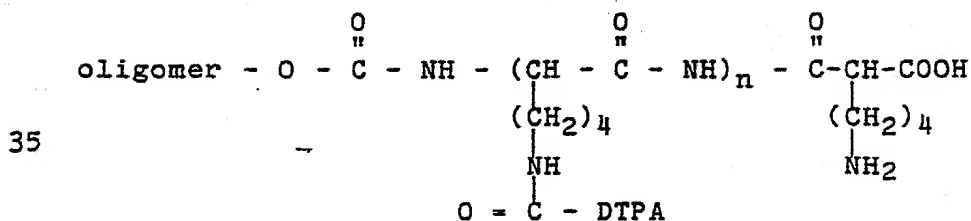
By varying the reaction excess of the DTPA or
the molecular size of polylysine used, it is possible
to construct conjugates with varying degrees of substi-
5   tution, size and charge. The ability to vary these
properties of the complex make it possible to design
the use of the compound in various applications.

EXAMPLE 4
10   Synthesis of DTPA Derivatives of
DNA Methylphosphonates
The chemical synthesis of DNA methylphophon-
ates may be carried out using a modification of the
phosphochloridite method of Letsinger (Letsinger et
15   al., J. Am. Chem. Soc. (1975) 97:3278; Letsinger and
Lunsford, J. Am. Chem. Soc. (1976) 98:3655-3661; Tanaka
and Letsinger, Nucl. Acids Res. (1982) 10:3249).
However, the preferred method and the one used in this
example uses methyl phosphonamidites (Applied
20   Biosystems, Foster City, CA). The method for
performing the synthesis uses exactly the same steps
and reaction times as in conventional DNA synthesis,
with the exception that THF rather than acetonitrile is
used to dissolve the phosphonamidites due to their
25   reduced solubility in the latter. Normal cycle times
using this procedure are 15 minutes with condensation
efficiencies of >94% (as judged by trityl release).
The ultimate base may be added as the cyanoethyl phos-
photriester which yields, upon cleavage in base, a 5'
30   terminal phosphodiester. This step makes it possible
to radiolabel the oligonucleotide, purify and sequence
the product using gel electrophoresis at intermediate
stages of preparation (Narang et al, Can. J. Biochem.
(1975) 53:342-394; Miller et al., Nucl. Acids Res.
35   (1983) 11:6225-6242).
An amine terminated linker arm is then added
as follows. Trityl is removed as before and the resin

treated with 0.2 M Aminolink (Applied Biosystems,
Foster City, CA) dissolved in dry acetonitrile contain-
ing 0.2M dimethylaminopyrodine for 5 minutes. The
linker arm oligonucleotide is then oxidized in iodine
5   and washed in acetonitrile as above. Capping with
acetic anhydride is not performed since any deblocked
primary amine would be modified to the base stable
acetamide and thus unavailable for further reaction.

At the end of the synthesis, the amine termi-
10   nated linker arm methylphosphonate oligomer is base
deblocked as follows. The resin containing the DNA is
removed from the column and placed in a water jacketed
column and incubated in 1-2 ml phenol:ethylene diamine
(4:1) for 10 hours at 40°C. At the end of the incuba-
15   tion in phenol:ethylene diamine, the resin is washed
free of the phenol reagent and released base protecting
groups using methanol, water, methanol and methylene
chloride in succession. After drying in a stream of
nitrogen, the intact, base-deblocked chains are cleaved
20   from the support using EDA:ethanol (1:1) or brief
treatment with room temperature ammonium hydroxide.

Purification of the amine terminated DNA
methylphosphonate is then performed as follows. The
material is first lyophilized several times from 50%
25   aqueous ethanol and purified via reversed phase HPLC C-
8 silica columns eluting 5 to 50% acetonitrile/25 mM
ammonium acetate, pH 6.8 in a linear gradient. Amine
containing fractions as determined by fluorescamine
reactivity are pooled and the product recovered by
30   drying in vacuo and further purified by ion-exchange
HPLC on Nucleogen DEAE 60-7 eluting 20% aceonitrile/25
mM ammonium acetate, pH 6.5.

The purified product is then converted to the
DTPA derivative as in EXAMPLE 1. Purification of the
35   complex is then effected as previously described.
Alternatively, unbound oligonucleotide is removed by
gel filtration on Sephadex G-100 or HPGFC on TSK
G4000SW eluting 10 mM Tris, pH 7.5.

The structure of the final product of this
procedure is illustrated as:

$$\text{oligomer MP} - \overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{P}} - O - (CH_2)_2 - NH - \overset{\overset{\displaystyle O}{\|}}{C} - DTPA$$
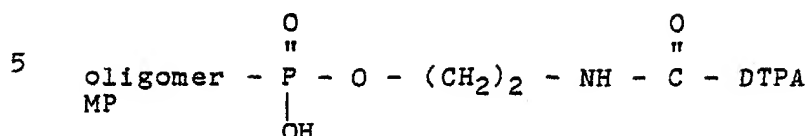
EXAMPLE 5

Synthesis of DTPA Derivatives of DNA
Ethyltriesters Using the Phosphoramidite Approach

The synthesis of the title compound triesters
is performed according to the method of Zon and cowork-
ers (Gallo et al., Nucl. Acids Res. (1986) 14:7405;
Summers et al., Nucl. Acids Res. (1986) 14:7421-7436).
The method of synthesis is similar to that which is
used for in situ production with ethyl triesters as
described by Letsinger (Letsinger et al., J. Am. Chem.
Soc. (1975) 97:3278; Letsinger and Lunsford, J. Am.
Chem. Soc. (1976) 98:3655-3661; Tanaka and Letsinger,
Nucl. Acids Res. (1982) 10:3249). In brief, fully
blocked dimethoxytrityl nucleosides are derived by
repeated lyophilization from benzene, dissolved in
anhydrous acetonitrile/2,6-lutidine (8:2) and added
dropwise to a stirred solution of chloro diisopropyl-
amino ethoxyphosphine in the same solvent at -70°C.
The product is recovered by aqueous extraction, in
vacuo drying and silica gel chromatography.

The chemical synthesis of DNA can be carried
out using slight modifications of the conventional
phosphoramidite methods. In this technique, nucleoside
phosphoramidites dissolved in anhydrous acetonitrile
are mixed with tetrazole and sequentially coupled to
the 5' hydroxy terminal nucleoside bound to CPG.
Nucleoside addition is followed by capping of unreacted
5' hydroxyls with acetic anhydride, iodine oxidation,
and 5' detritylation in trichloroacetic acid-methylene

chloride. The resin bound oligomer is then dried by extensive washing in anhydrous acetonitrile and the process repeated. Normal cycle times using this procedure are 17 minutes with condensation efficiencies of

5 >96% (as judged by trityl release). The terminal residue is conventionally added as a diester in order to facilitate radiolabeling and purification. The 5' terminal trityl group is left if HPLC purification is desired, but generally the 5' terminal trityl is

10 removed and the aminolink procedure described in EXAMPLE 1 is used.

At the end of the synthesis, the fully blocked product is base-deblocked as follows. The resin containing the fully protected DNA is removed from the

15 column and placed in a water jacketed chromatography column. The resin is then incubated in 1-2 ml phenol:ethylene diamine (4:1) for 10 hours at 40°C. At the end of the incubation in phenol:ethylene diamine, the resin is washed free of the phenol reagent and

20 released base protecting groups using methanol, water, methanol and methylene chloride. After drying in a stream of nitrogen, the intact, base-deblocked chains are cleaved from the support using EDA:ethanol (1:1) or brief treatment with room temperature ammonium

25 hydroxide.

Purification of the aminolink DNA ethyl-triester product is then performed as follows. The material is first lyophilized several times from 50% aqueous ethanol and purified via reversed phase HPLC C-

30 8 silica columns eluting 5 to 50% acetonitrile/25 mM ammonium acetate, pH 6.8 in a linear gradient. Amine containing fractions as determined by fluorescamine reactivity are pooled and the product recovered by drying in vacuo and further purified by ion-exchange

35 HPLC on Nucleogen DEAE 60-7 eluting 20% acetonitrile/25 mM ammonium acetate, pH 6.5.

The product oligonucleotide is then suitable
for coupling to DTPA and purification by the techniques
previously described.

The structure of the final product of this
procedure is illustrated as:

$$\text{oligomer} \atop \text{ETE} - \overset{\overset{\displaystyle O}{\|}}{P} - \underset{\underset{\displaystyle OH}{|}}{O} - (CH_2)_2 - NH - \overset{\overset{\displaystyle O}{\|}}{C} - DTPA$$
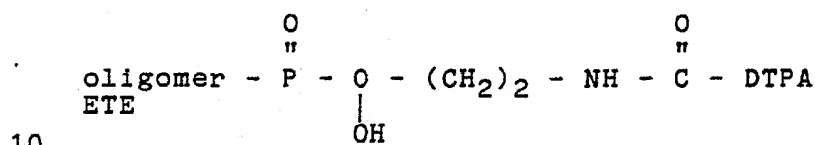
EXAMPLE 6
Synthesis of DTPA Derivatives of DNA Alkyl
and Aryltriesters Using the
Phosphate Triester Approach

A preferred method for the production of the
oligonucleotide triesters of variable alkane chain
length is via conventional phosphate triester chemistry
to synthesize the desired sequences as the p-
chlorophenyl phosphate triesters. Upon completion of
the synthesis, the fully protected oligonucleotide
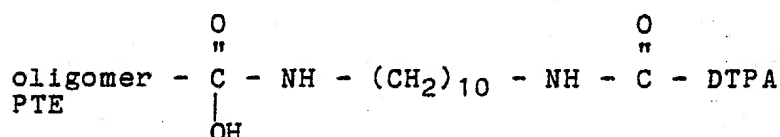chlorophenyltriesters bound to the synthesis support
are subjected to ester exchange in the presence of
tetrabutylammonium fluoride and the desired alcohol.
This basic method for the construction of DNA oligo-
nucleotides is classical DNA synthesis chemistry and
presents no problems. The essential chemistry is well
described (Gait, Oligonucleotide Synthesis: A Practical
Approach IRL Press, Washington, D.C. (1984)) and can be
used with little modification. An alternative phos-
phite based chemistry which is much more rapid and
gives equivalent yields is set forth below.

The chemical synthesis of DNA p- or o-
chlorophenyl phosphotriesters was carried out using a
modification of the phosphochloridite method of
Letsinger (Letsinger et al., J. Am. Chem. Soc. (1975)
97:3278; Letsinger and Lunsford, J. Am. Chem. Soc.

(1976) 98:3655-3661; Tanaka and Letsinger, Nucl. Acids
Res. (1982) 10:3249). A programmable, automated DNA
synthesizer used for phosphomonochloridite based
syntheses (Alvarado-Urbina et al., Science (1981)
5   214:270-273.

Fully blocked and carefully dried nucleosides
dissolved in anhydrous acetonitrile, 2,6-lutidine and
activated in situ with chlorophenoxydichlorophosphine
are sequentially added to the 5' hydroxy terminal
10   nucleotide of the growing DNA chain bound to controlled
pore glass supports via a succinate spacer (Matteucci
and Caruthers, Tetrahedron Lett. (1980) 21:719-722).
Derivatized glass supports, fully block nucleosides and
other synthesis reagents are commercially available
15   through Applied Biosystems (San Francisco, CA) or
American Bionuclear (Emeryville, CA). Nucleoside
addition is followed by capping of unreacted 5'
hydroxyls with acetic anhydride, iodine oxidation, and
5' detritylation in trichloroacetic acid-methylene
20   chloride.

The resin bound oligomer chlorophenyltriester
is then dried by extensive washing in anhydrous aceto-
nitrile and the process repeated. Normal cycle times
using this procedure are 13 minutes with condensation
25   efficiencies of >92% (as judged by trityl release).
The ultimate base may be added as a methyl phospho-
triester which yields, upon cleavage in base, a 5'
terminal phosphodiester. This step makes it possible
to radiolabel the oligonucleotide and to purify and
30   sequence the product using gel electrophoresis (Narang
et al., Can. J. Biochem. (1975) 53:392-394; Miller et
al., Nucl. Acids Res. (1983) 1:6225-6242).

The fully blocked material bound to the
synthesis support is then subjected to ester exchange
35   in the presence of tetrabutylammonium fluoride (TBAF)
and the desired alcohol. This method yields rapid and
quantitative alcohol exchange. The reaction is com-

29

plete within 20 minutes for most aryl and alkyl alco-
hols which are capable of forming stable products. With
any alcohol, the presence of trace amounts of water can
affect the overall yield. Thus, care must be taken to
used anhydrous alcohols at this step.

In this example, anhydrous n-propanol is used
to dissolve TBAF to a final concentration of 0.2 M.
The solution is then percolated slowly over the resin
containing the oligomer chlorophenyl triester and
allowed to react for about 1 hour at room temperature.
The resin is then washed with methanol and acetonitrile
and dried under a stream of dry argon. Amine linker
arm addition, deblocking and purification are then
effected as in EXAMPLE 2. DTPA conjugation is then
performed as in EXAMPLE 1. The final yield of conju-
gate is about 10% of the starting equivalents of
nucleoside resin used.

The structure of the final product is illu-
strated as:

$$\text{oligomer PTE} - \overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle OH}{|}}{C}} - NH - (CH_2)_{10} - NH - \overset{\overset{\displaystyle O}{\|}}{C} - DTPA$$

EXAMPLE 7

Effect of DTPA Conjugates on the Synthesis
of Hemoglobin in Mouse MEL Cells

The effectiveness of oligomer DTPA conjugate
mediated HART (Paterson et al., Proc. Natl. Acad. Sci.
USA (1977) 74:4370; Hastie and Held, Proc. Natl. Acad.
Sci. USA (1978) 75:1217-1221) was determined in
cultured cells incubated in the presence or absence of
the oligomer. The cells used were Friend murine
erythroleukemia (MEL) cells which can be induced to
synthesize hemoglobin by a variety of agents including
DMSO and butyric acid (cf. Gusella and Houseman, Cell

(1976) $\underline{8}$:263-269). Friend leukemia cells were grown in culture using well known techniques in a CO_2 incubator. Hemoglobin synthesis was induced using 1.5% DMSO.

5   Induced cells expressing hemoglobin were visualized by benzidine treatment which stains globin producing cells blue (Leder et al., Science (1975) 190:893). Cells were exposed to selected oligonucleotides and DTPA conjugate at concentrations ranging from 0.1 μM to 50 μM during a 4- to 5-day induction period.
10 Controls included mock-treated cells and cells treated with unmodified oligomers of the same sequence. Treated cells were scored for globin production based on staining intensity and the results compared to controls. Cell death or damage due to treatment was
15 scored by Trypan blue exclusion.

     The following Table (Table I) indicates the specific sequences synthesized.

TABLE I

DNA SEQUENCES SYNTHESIZED AND CONJUGATED
FOR USE IN CELL CULTURE EXPERIMENTS

Probes Synthesized Antisense to Mouse Beta-globin mRNA	% GC	Sequence (3' to 5')
MBG 15 methylphosphonate	60%	g tac cac gtg gac tG
MBG 15 methylphosphonate-C_2amine	60%	g tac cac gtg gac tGp-O-$(CH_2)_2$-NH_2
MBG 15 methylphosphonate DTPA conjugate	60%	g tac cac gtg gac tGp-O-$(CH_2)_2$-NH-C(O)-DTPA
MBG 20 antisense	55%	G TAC CAC GTG CAC TGA CTA C
MBG 20 antisense C_2-amine	55%	G TAC CAC GTG CAC TGA CTA Cp-O-$(CH_2)_2$-NH_2
MBG 20 antisense C_6-amine	55%	G TAC CAC GTG GAC TGA CTA C-O-(CO)-NH-$(CH_2)_6$-NH_2
MBG 20 antisense C_2-amine DTPA conjugate	55%	G TAC CAC GTG GAC TGA CTA Cp-O-$(CH_2)_2$-NH-C(O)-DTPA

a) Lower case letters represent nucleosides coupled to the 3'
adjacent nucleoside via a methylphosphonate linkage. Upper case
letters represent 3' adjacent normal phosphodiester linkage. C_2
derivatives are formed from the condensation of ethanolamine with
a 5' terminal phosphate via an ester linkage. C_6 and C_{10}
derivatives are the corresponding diamines coupled via an alkyl
carbamate linkage to the 5' terminal hydroxyl. DTPA represents
5   diethylenetriamine pentaacetic acid.

The data in the following Table (Table II)
show that DTPA conjugated oligomers were approximately
500 times more effective than control oligomers with or
10 without the amine linker attached. Significant cyto-
toxicity was observed only at concentrations above
10 μM, about 100 times the minimum dose for a
significant effect.

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TABLE II

The Effect of Cleaver Conjugate Oligonucleotides in Preventing the Synthesis of Hemoglobin in Cultured Cells

Treatment [1]	Conc.	% Viable Cells	% Inhibition Globin+ Cells
Experiment #1 - Effect Compared to Other Constructs.			
DMSO Control		46%	0%
MBG-20 Antisense	50 µM	50%	41%
MBG-20-C_2amine	50 µM	61%	41%
MBG-20-DTPA Conjugate	50 µM	0%	100%
Experiment #2 - Concentration Dependence of DTPA Conjugate			
DMSO Control	1.5%	65%	0%
Solvent Control		n.d	0%
EDTA Control	1 µM	65%	0%
MBG-20-DTPA Conjugate	1 µM	50%	98%
		57%	96%
	500 nM	34%	95%
		14%	96%
	300 nM	65%	84%
		65%	84%
	200 nM	78%	58%
	100 nM	69%	56%
		76%	60%
	10 nm	67%	47%
		74%	61%
	1 nM	70%	41%
	0.1 nM	72%	2%
	none	nd	0%

Table II
(continued)

Experiment #3 - Effect of Methylphosphonate DTPA Conjugate

Treatment [1]	Conc.	% Viable Cells	% Inhibition
DMSO Control		50%	0%
MBG 15 methyl-phosphonate DTPA Conjugate	100 nM	50%	48%
	10 nM	51%	43%
	1 nM	54%	37%
	0.1 nM	54%	37%

[1] C_2 derivatives are formed from the condensation of ethanolamine with a 5' terminal phsophate via an ester linkage. DTPA represents diethylenetriamine pentaacetic acid.

It is evident from the above results, that conjugates of a chelating agent and an oligonucleotide sequence may be used to preferentially inhibit the expression of a sequence in a viable cell. In this manner, cells can be modified in a variety of ways, so as to change the phenotype or to selectively kill cells. The conjugate is stable and does not require that a metal be non-covalently bound to the chelating agent prior to use in order to achieve effectiveness. In addition, the subject compositions can be used in vivo or in vitro, allowing for selection of cells, enhancing activity of particular cells, reducing activity of particular cells, or permitting selection of a particular class of cells. A variety of conjugates can be produced, which will have long half lives, so as to be able to provide for destruction of a large number of RNA sequences for each molecule conjugate.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention

pertains. All publications and patent applications are
herein incorporated by reference to the same extent as if
each individual publication or patent application was
specifically and individually indicated to be incorporated
5 by reference.

      Although the foregoing invention has been described
in some detail by way of illustration and example for
purposes of clarity of understanding, it will be obvious
that certain changes and modifications may be practiced
10 within the scope of the appended claims.

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WHAT IS CLAIMED IS:

       1.   A method of inhibiting the utilization of RNA in a cellular process, said method comprising:

       contacting cells with a composition comprising (1) a hybridizing sequence capable of hybridizing with an RNA sequence; joined to (2) a metal-ion free chelating agent capable of chelating a metal ion, said metal ion capable of initiating an intracellular reaction resulting in the inactivation of said RNA sequence,

       whereby said composition enters said cell and inactivates said RNA sequence.

       2.   A method according to Claim 1, wherein said hybridizing sequence is at least 50 number percent G + C.

       3.   A method according to Claim 1, wherein said chelating agent is a polyamino-polycarboxylic acid chelating agent.

       4.   A method according to Claim 3, wherein said chelating agent is an alkylenepolyamine polycarboxymethylene chelating agent.

       5.   A method according to Claim 1, wherein said chelating agent is linked to said hybridizing sequence through a linking chain of from 4 to 16 atoms in the chain.

       6.   A method according to Claim 1, wherein said cell is part of a mammalian host.

       7.   A method according to Claim 1, wherein said composition further comprises a moiety imparting amphiphilic properties to said composition, said moiety comprising at least 6 carbon atoms and hydrophobic or amphiphilic.

　　　　　　　8.　　A method of inhibiting the utilization of mRNA in a cellular process, said method comprising:

　　　　　　　contacting cells with a composition comprising (1) a hybridizing sequence capable of hybridizing with an mRNA

5　　sequence in said cells; joined to (2) a metal-ion free polyamino-polycarboxylic acid chelating agent capable of chelating a metal ion, said metal ion capable of initiating an intracellular reaction resulting in the inactivation of said RNA sequence,

10　　　　　　　whereby said composition enters said cell and inactivates said mRNA sequence.

　　　　　　　9.　　A method according to Claim 8, wherein said chelating agent is diethylene triamine pentaacetic acid.

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　　　　　　　10.　　A method according to Claim 9, wherein said chelating agent is joined to said hybridizing sequence through a linking chain of from 4 to 16 atoms in the chain and through a amide functionality.

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　　　　　　　11.　　A method according to Claim 8, wherein said hybridizing sequence of is a polyphosphate.

　　　　　　　12.　　A method according to Claim 8, wherein said
25　　hybridizing sequence is a polyphosphonate.

　　　　　　　13.　　A method according to Claim 8, wherein said hybridizing sequence has from about 8 to 30 units.

30　　　　　　　14.　　A method according to Claim 8, wherein said composition further comprises a moiety imparting amphiphilic properties to said composition, said moiety comprising at least 6 carbon atoms and hydrophobic or amphiphilic.

35　　　　　　　15.　　A composition comprising a hybridizing sequence comprising a total of from about 8 to 30 nucleosides and capable of hybridizing to an RNA sequence

covalently joined through a linking chain of from about 4 to 16 atoms in the chain to a polyamino-polycarboxylic acid chelating agent, said linking arm bonded to a sugar of said nucleoside.

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16.   A composition according to Claim 15, wherein said linking arm is bonded to said sugar through an ester linkage.

10   17.   A composition according to Claim 16, wherein said ester linkage is to a 5' hydroxyl of a sugar of the terminal unit of said hybrididizing sequence.

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I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12N 05/00; A61K 31/00
U.S. C1. 435/240.2, 514/44

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	435/6,41,91,240.2; 935/78; 514/44

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

Computer searched CAS (1967-1989), Biosis (1969-1989), APS (1975-1989). Terms: chelate, diethylene triamine pentaacidic acid, transport

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	US, A, 4707352 (Stavrianopoulos) 17 November 1987. See entire document.	1-17
Y	US, A, 4508625 (Graham) 02 April 1985. See entire document.	1-17
Y	Suzuki et al, An Introduction to Genetic Analysis, published 1986, by W.H. Freeman and Company (New York), see pages 191-193.	2

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 February 1989	19 APR 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	Lori Y. Beardell

Form PCT/ISA/210 (second sheet) (Rev.11-87)